An environmentally relevant BDE47 metabolite is highly toxic in zebrafish and has dramatic effects on metabolism *in vitro*

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Introduction

Polybrominated diphenylethers (PBDEs) seem to be only mildly toxic at high concentrations in rodents and fish so it is unclear whether these compounds provide a threat to the environment or to human health (Darnerud 2003;Birnbaum and Staskal 2004). Of all the PBDE congeners found in the environment, 2,2',4,4'-tetrabromodiphenylether (BDE47) is the most abundant and probably persistent. BDE47 is toxic to rats and mice at high concentrations (Darnerud 2003) and has been shown to undergo phase I metabolism in vertebrates, as studies in both rodents and fish have shown the formation of methoxylated (MeO-) and hydroxylated (OH-) BDE47 metabolites after administration of a single dose of BDE47 (Hakk and Letcher 2003;Birnbaum and Staskal 2004). Furthermore, both MeO- and OH-BDE47 have been detected in the blood of fish, wildlife and humans (Hovander et al. 2002;Marsh et al. 2004;Sinkkonen et al. 2004; Valters et al. 2005; Verreault et al. 2005).

The hydroxylated BDE47 metabolite 6-OH-BDE47 is a major metabolite within the pool of hydroxylated BDEs retained in biological systems (Valters et al. 2005). Importantly, this compound is structurally identical to a naturally occurring chemical. Red algae (*Ceramium tenuicorne*) and blue mussels (*Mytilus edulis*) in the Baltic Sea have been shown to produce both hydroxylated and methoxylated BDE47 without the presence of BDE47 itself (Malmvärn et al. 2005). Though *in vitro* studies have demonstrated the potential endocrine disrupting and cytotoxic properties of 6-OH- BDE47 (Hamers et al. 2006; Canton et al. 2006; Canton et al. 2005) nothing is known of the *in vivo* toxicity of this both naturally occurring and anthropogenic BDE metabolite.

The main goal of this study was to determine the toxicity of 6-OH-BDE47, 6-MeO-BDE47 and BDE47 and to investigate possible modes of action. We show here that 6-OH-BDE47 is acutely toxic to zebrafish embryos, whereas 6-MeO-BDE47 and BDE47 are not. Additional *in vitro* studies indicate that 6-OH-BDE47 caused dramatic changes in cellular metabolism in human hepatocyte derived (HepG2) cancer cells. Taken together, these results provide the first evidence of toxicity of a BDE metabolite of the most relevant BDE in our environment.

Materials and Methods

Compounds
All compounds were prepared by dissolving them in dimethylsulfoxide (DMSO) (Acros, Belgium). The compound 2,2',4,4',-tetrabromodiphenylether (BDE47) was purified and 2,2',4,4',-6 hydroxytetrabromodiphenylether (6-OH-BDE47) and 2,2',4,4',-6 methoxytetrabromodiphenylether (6-MeO-BDE47) were synthesised in the group. Coppersulfate

(Cu_2SO_4), Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and rotenone were obtained from Sigma (The Netherlands).

Zebrafish toxicity experiments

Wild type zebrafish were obtained from Ruinemans, Montfoort, The Netherlands and maintained under standard conditions. For embryotoxicity experiments, eggs were collected and fertilization and quality were assessed under a stereo microscope (M7.5; Leica, The Netherlands). After three hours 25 – 30 developing eggs were exposed in 50 ml Dutch Standard Water (DSW; 100 mgL^{-1} $NaHC0_3$, 20 mgL^{-1} $KHCO_3$, 180 mgL^{-1} $MgSO_4.7H_2O$) at 28°C in 100 ml beaker glasses containing the appropriately diluted compounds in DMSO (Acros, Belgium). All experiments were conducted with approval of a Dutch animal ethical commission.

Cell viability assays

Human hepatocyte-derived cancer (HepG2) cells were maintained in DMEM F12 culture medium (Gibco, The Netherlands) containing 10% fetal bovine serum. For cell viability by cellular ATP content, 2.5 x 10^4 HepG2 cells were plated in triplicate in 96 well tissue culture plates (Nunc, The Netherlands) in complete growth media. The next day ATP was measured using a cellular ATP detection kit (Biothema, Denmark) according to the manufacturers' instructions. Luciferase activity was measured using a Lucy2 luminometer (Anthos, Austria) immediately after addition of the appropriate reagents.

Cell viability was also measured as the metabolic capacity to reduce blue resazurin into pink resorufin basically as described before (O'Brien et al. 2000). In brief, HepG2 cells were exposed to 6-OH-BDE47, 6MeO-BDE47, BDE47 for two hours and FCCP, rotenone and Cu_2SO_4 was used as positive control. Metabolic capacity was determined spectrophotometrically (SpectraMAX 340 PC) as a decrease of the blue-colored resazurin (k ¼ 600 nm) in the assay medium. All experiments were repeated at least three times and the decrease in resazurin was expressed as percentage of control.

Respiration assay

Cellular respiration was measured using a fluorescence based assay basically according to the manufacturers' instructions (Luxcel Biosciences, Ireland). In brief, human hepatocyte-derived cancer (HepG2) cells were maintained as described above. For measuring respiration, cells were plated in triplicate in 96 well tissue culture plates (Nunc, The Netherlands) at 5.0 x 10^5 cells per well in growth medium. The next day, the medium was aspirated and replaced by pre-warmed complete medium containing 0.15 μM of oxygen probe, type A65N (Luxcel Biosciences, Ireland) and the appropriate compound or solvent (DMSO) diluted 1:1000 directly into the medium. All wells were sealed with 100 μl of pre-warmed mineral oil (Sigma, The Netherlands) and read kinetically every 2 minutes, 20 times at 37 °C on a time resolved fluorescence plate reader (Wallac Victor^2, The Netherlands). Measurement settings were: excitation / emission: 340 nm/642 nm; counting delay: 30 μs; counting window: 100 μs. Experiments were carried out in at least threefold.

Results and Discussion

To gain more insight in the embryo toxic effects of 2,2',6,6',-tetrabromodiphenylether (BDE47), its major hydroxylated metabolite 2,2,6,6,-6 hydroxytetrabromodiphenylether (6-OH-BDE47) and its methoxylated isomer 2,2,6,6,-6 methoxytetrabromodiphenylether (6-MeO-BDE47), zebrafish embryos were exposed from 3 hpf (hours post fertilization) for the first 72 hrs of development. For BDE47 and 6-MeO-BDE47, no toxic or teratogenic effects were observed at

concentrations of up to 10 µM (data not shown). Exposure to 25-50 nM 6-OH-BDE47 caused a wide range of developmental defects while 100 nM caused acute toxicity with embryos undergoing developmental arrest around the 18 somite stage. The effects on development of 6-OH-BDE47 were dose related and expressed as the percentage of total effected embryos (figure1). Preliminary data indicated that 6-OH-BDE47 is acutely toxic in adult zebrafish at concentrations of 500 nM and 1 µM 6-OH-BDE47 within the first hour of exposure. These findings suggest a mode of action (MOA) for acute toxicity in zebrafish independent of life stage.

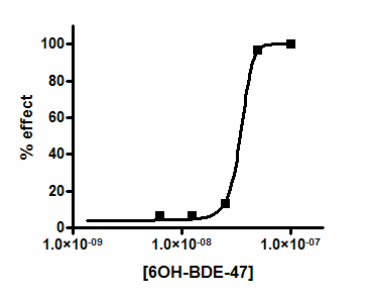

Figure 1. Toxic effects of 6-OH-BDE47 on zebrafish embryos. Zebrafish embryos were exposed in triplicate to different concentrations 6-OH-BDE47Abnormal development was scored blind at 72 hours post fertilization for 25 to 50 embyos in triplicate.

Due to the acute toxic nature of 6-OH-BDE47 in both zebrafish embryos and adult zebrafish, we investigated cytotoxicity with two different assays; reduction in ATP content and the conversion of rezasurin in to resorufin in human hepatocyte derived (HepG2) cancer cells (figure2a). ATP was measured in a luciferase assay and compared to the mean ATP concentration in untreated cells. We observed a reduction in the total amount of ATP after incubation with 6-OH-BDE47, and the cytotoxic agents FCCP and rotenone while no reduction was observed with Cu_2SO_4 or with BDE47 and 6-MeO-BDE47. Additionally, we assessed the metabolic capacity of HepG2 cells to reduce blue resazurin into pink resorufin (O'Brien et al. 2000). Cu_2SO_4 showed a clear decrease in this capacity while FCCP, rotenone, 6-OH-BDE47, BDE47 and 6-MeO-BDE47 did not show a significant reduction. Subsequently, we investigated changes in cellular respiration rates. In short, cells were exposed to FCCP, rotenone and 6-OH-BDE47 and cellular respiration was measured kinetically, using a fluorescent probe that is quenched by oxygen. Rotenone clearly showed inhibition of cellular respiration as shown by the decreased slope of fluorescence increase in figure2b, whereas FCCP and 6-OH-BDE47 showed a clear increase in respiration rate. These effects were not observed for either BDE47 or 6-MeO-BDE47 (data not shown).

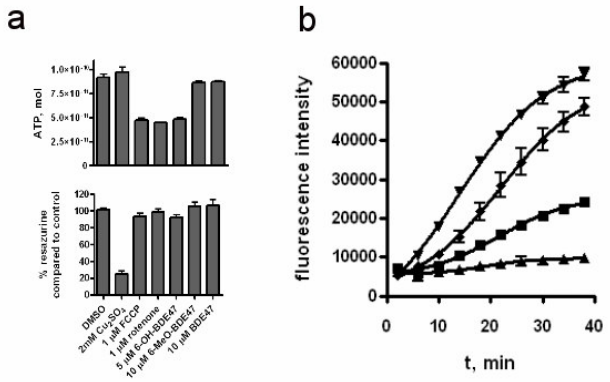

Figure 2. Metabolic changes in 6-OH-BDE47 exposed HepG2 cells. (a) HepG2 cells were exposed to DMSO; 2 mM Cu2SO4; 1 µM FCCP; 1 µM rotenone; 5 µM 6-OH-BDE47; 10 µM 6-MeO-BDE47 and 10 µM BDE47. (a) ATP is

expressed as the average total amount of ATP per well in moles ± standard error of the mean (SEM). Cytotoxicity was determined after a 2 hour exposure to the compounds in a resazurin assay. Cytotoxicity is expressed as the percentage of resazurin conversion in DMSO treated cells ± SEM. (b) Cell respiration was determined by the increase of fluorescence of an oxygen sensitive probe in time (t). Depicted is the absolute amount of fluorescence counts in time after exposure to 1 µM FCCP (▼); 1 µM rotenone (▲); 5 µM 6-OH-BDE47 (♦) or DMSO (■) of one representative experiment. The error bars represent the standard error of the mean for triplicates within the experiment.

In conclusion, we show here that 6-OH-BDE47 is acutely toxic in zebrafish embryos and in human cells. To our knowledge this is the first report of a toxic effect *in vivo* of a metabolite of the most relevant BDE (BDE47) in the environment. Furthermore we provide evidence that 6-OH-BDE47 causes a dramatic change in cellular metabolism. The possible mode of action of 6-OH-BDE47 is currently being investigated further. We strongly recommend continued research into the toxicity of phenolic BDEs of both natural and anthropogenic origin.

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